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13. ABSTRACT (Maximum 200 Words)

Circadian influence on breast tumorigenesis is well-documented by epidemiological studies and clinical data, although the molecular kinetics remain elusive. Work involving circadian clock genes and cell cycle components suggests not only an association between the two time-keeping systems, but also regulation of the cell cycle by the circadian clock. We provide further evidence indicating circadian clock control of the cell cycle in the breast cancer model. Over-expression of the clock gene, PERIOD-2 (PER2), induced significant growth inhibition of MCF-7 breast cancer cells. The antiproliferative effects of PER2 seemed attributable to the induction of apoptosis since over-expressing cells exhibited changes in cell morphology (rounding up and detachment) which are consistent with programmed cell death. Apoptosis was confirmed by a significant increase in PARP (poly(ADP-ribose)polymerase) cleavage, an indicator of activated caspases. In addition, there was a marked decrease in the protein expression of cyclin D1 with a concomitant up regulation of p21 expression. Studies are underway to determine the relationship between p53 and PER2 as a possible mechanism to explain growth inhibition and apoptosis. We are also examining the effects of PER2 over-expression on cell cycle distribution. Our data thus far suggest that PER2 functions as a tumor suppressor in breast tissue.

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INTRODUCTION:

Circadian rhythms are an evolutionarily conserved property that regulates hundreds of functions in the human body including sleep and wakefulness, body temperature, blood pressure, hormone production, digestive secretion and immune activity. Our health is severely affected by disruptions of these rhythms. For example, alterations of circadian pattern have been linked to insomnia, jet lag, depression, and are commonly observed among cancer patients (1,2).

In mammals, a master circadian pacemaker resides in the hypothalamic suprachiasmatic nuclei (SCN). The SCN consists of multiple, autonomous single cell circadian oscillators that take in photic cues via the retinohypothalamic tract to synchronize the organism with light/dark phases (3). The molecular basis of circadian timing involves a transcriptional-translational negative feedback loop that drives recurrent oscillations in the RNA and protein levels of eight core circadian clock genes (4). These genes include the three mammalian homologues of *period* (PER1, PER2, PER3), *clock* (CLK), *brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein 1* (BMAL1), *cryptochrome 1* (CRY1), and *cryptochrome 2* (CRY2). Clock genes are not only expressed in the SCN but in every tissue investigated to date and can be induced in cultured cells by serum shock or steroid hormones such as glucocorticoids (5,6). The SCN central clock and peripheral tissue clocks both regulate cell functions by controlling the expression of clock-controlled genes which are estimated to account for 2-10% of all mammalian genes. Most notable among the clock-controlled genes are those that encode key regulators of cell-cycle progression which include Cyclin D1, Cyclin A, Mdm-2, and c-Myc (7-10).

For many years now, it has been known that disruption of circadian rhythm increases the rate of tumorigenesis, but until recently no molecular evidence was available to explain this phenomenon. Breast cancer is especially susceptible to circadian alterations due to the fact that it is an endocrine responsive carcinoma and many hormones exhibit diurnal oscillations (1). Several epidemiological studies have revealed a role for the circadian clock in breast cancer development. A Danish study investigating 30- to 54-year old women concludes that women who work predominantly at night show an increased risk of mammary tumorigenesis. The breast cancer risk increases greater still with the number of years or hours that individuals spend working at night (11). Similar results are described by Schernhammer *et al.* (12) who examined 78,562 nurses that often alternate between day and night shifts and Davis *et al.* (13) who investigated sleeping habits and bedroom lighting of healthy individuals. All of these studies emphasize that circadian rhythm interruption increases an individual's risk for the development of breast cancer.

In tumor-bearing animals and cancer patients, circadian disruption not only increases the risk of tumor development, but also accelerates cancer progression and is associated with poor prognosis and outcome. Complete ablation of the SCN in mice results in loss of circadian rhythm as well as a two- to three-fold increase in malignant growth when compared to controls leading to significant reductions in survival time (14). Carcinoma- or sarcoma-bearing rats show an increase in tumor growth and a reduction in survival time when subjected to alternating photoperiods (15). Using salivary cortisol levels as a measure of circadian rhythm, metastatic breast cancer patients who had lost normal diurnal salivary cortisol variation exhibit earlier mortality (16).

The mechanism by which the circadian clock affects tumor growth is not fully understood; however, PER2 has recently been suggested to function as a tumor suppressor. While performing functional studies, Fu *et al.* observed spontaneous lymphomas, teratomas, and salivary-gland hyperplasia in *per2* knockout mice at six months of age (7). Thirty per cent of the mutant mice died before the age of 16 months. The animals were also more sensitive to gamma radiation—*per2* mutant thymocytes show a G2/M specific resistance to radiation induced apoptosis. In fact, *per2* disruption abolishes the response of all core circadian genes to gamma radiation whereas in wild-type mice the clock genes are induced rapidly suggesting they could be involved in DNA damage response. Many cell

cycle and checkpoint components are dysregulated in these *per2* mutant mice such as *cyclin D1*, *cyclin A*, *mdm-2*, *gadd45α*, and *c-myc*. Interestingly, the transcription of the oncogene, *c-myc*, was circadian in wild-type liver; but in the mutants the expression was shifted and dramatically increased. Moreover, *in vitro* experiments revealed that *c-myc* expression is directly under the control of clock genes. Based on these data we hypothesized *that the clock gene, per2, is expressed in normal human breast and at a reduced level in breast cancer altering the cell cycle, cell growth and possibly apoptosis.*

Perturbations in clock genes such as per2 could possibly lead to the progression of malignancies by diminishing the length of G1 phase of the cell cycle, reducing the ability of a cell to respond to genetic damage, and preventing apoptosis. We intend to further characterize the role of Per2 in cell cycle control and cell growth. If our hypothesis is correct, specific treatments could be designed to alleviate Per2 dysfunction. Our results may also help to enhance the therapeutic index of cancer drugs and/or their antitumor efficacy by administering them when Per2 levels are the highest and cell are most sensitive to apoptosis, thus contributing to the overall improved outcome of breast cancer patients.

BODY:

Our preliminary studies and those of others suggested that Per2 might function as a potential tumor suppressor gene in a variety of tissues including the breast. However, the specific studies defining if Per2 is differentially expressed in malignant vs. normal breast epithelium have not been conducted. As well, several studies have indirectly implicated that Per2 expression could repress cell proliferation and cell cycle, but the definitive studies demonstrating the direct suppression of breast tumor cell cycle by Per2 expression have not been conducted. Thus, we have set out to characterize per2 expression in normal vs. malignant breast epithelial cells, to define the importance of per2 expression on cell cycle progression and the ability of per2 in induce apoptosis in breast tumor cells. To accomplish these goals we proposed the following objectives for this one year proposal.

Objectives:

1. To measure *per2* expression at the mRNA and protein levels in normal breast tissue versus cancerous breast tissue the following techniques will be employed:
   a. Laser Capture Microdissection (LCM) to isolate tumor cells and normal epithelial cells from primary tumors and reduction mammoplasty specimens.
   b. Real Time-PCR (RT-PCR) of Per2 mRNA levels in normal breast epithelial cells, breast tumor cells from primary tumors, and normal breast epithelium and breast cancer cell lines.
   c. Western blot analysis of Per2 protein levels in normal breast epithelial cells, breast tumor cells from primary tumors, and normal breast epithelium and breast cancer cell lines.
2. To characterize the effect of Per2 on the cell cycle by:
   a. Flow cytometric analysis of Per2 overexpression in MCF-7 and MDA-MB-231 cells.
   b. Flow cytometric analysis of Per2 underexpression in MCF-7 and MDA-MB-231 cells.
3. To define the molecular mechanisms by which Per2 affects the cell cycle using the following strategies:
   a. Overexpressing Per2 in MCF-7 and MDA-MB-231 cells and examining mRNA expression of *cyclin D1*, *mdm2*, and *c-myc* by Northern blot analysis.

To accomplish the studies proposed in these Objectives we proposed the following Statement of Work:

Months 1-4:

To measure per2 expression at the mRNA and protein levels by real time-PCR and Western blot analysis in normal breast tissue vs. cancerous breast tissue by employing Laser Capture Microdisection (LCM) to isolate tumor cells and normal epithelial cells from primary tumors and reduction mammoplasty specimens.

Months 4-8:

To characterize the effect of perr2 on the cell cycle using flow cytometric analysis of Per2 overexpression in MCF-7 and MDA-MB-231 cells. We will also conduct flow cytometric analysis of per2 underexpression in MCF-7 and MDA-MB-231 cells.

Months: 9-12:

To define the molecular mechanisms by which per2 affects the cell cycle by overexpressing Per2 in MCF-7 and MDA-MB-231 cells and examining mRNA or protein expression of *cyclin D1, mdm2 and c-myc.*

Per2 mRNA expression in normal human breast epithelium vs.malignant breast tumor cells:

For these studies we obtained breast tumor biopsy specimens from the Tulane Cancer Center Tumor Bank. These tissue blocks are kept frozen in a –80° C freezer and contain both malignant epithelium as well as normal mammary epithelial and stromal cells. Estrogen and progesterone receptor status was available on the tumor samples employed for these analyses. Unfortunately, real-time PCR analysis has not been completed at this point but is well underway. However, standard semi-quantitative RT-PCR has been completed for Per2 analysis of normal vs. malignant beast epithelial cells from breast tumor samples. Using LCM technology (Department of Urology, Tulane Medical School) we were able to isolate mammary tumor cells or normal mammary epithelial cells. 10-20 cells of each type were pooled and mRNA isolated by standard small sample RNA isolation procedures. RT-PCR analysis of normal breast epithelial cells showed a relatively strong expression of Per2 mRNA in 5 different samples, as shown in Figure 1. Analysis of Per2 mRNA levels in malignant breast epithelial cells demonstrates either a complete absence of Per2 or a reduction (2-3 fold) of Per2 mRNA levels as compared to the levels seen in the normal breast epithelial cells. These data demonstrate that Per2 expression is greatly reduced or completely absent in malignant breast epithelial cells as compared to normal non-malignant breast epithelial cells from corresponding tumor samples.

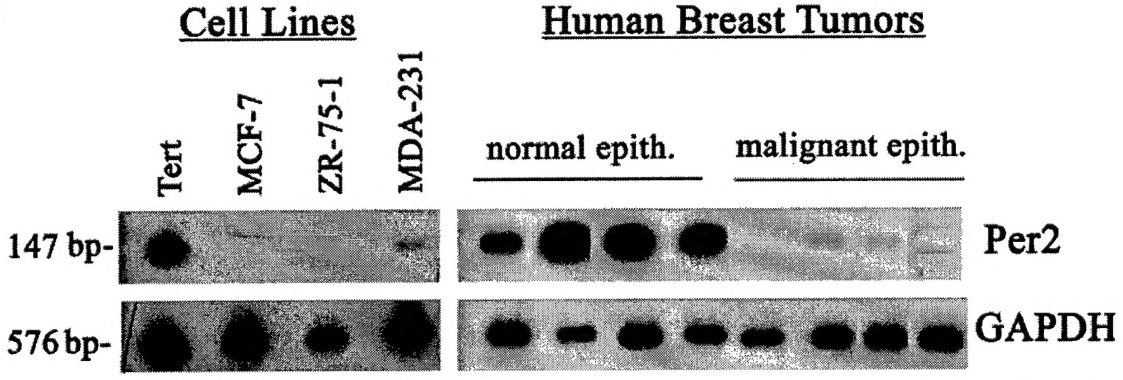


Figure 1. RT-PCR analysis of Per2 mRNA expression in normal vs. malignant breast epithelium from breast tumor samples.

Effects of Per2 on MCF-7 cell proliferation and cell cycle:

Our studies have demonstrated that the Per2 gene is expressed at both the mRNA and protein levels in normal mammary epithelium and non-malignant mammary epithelial breast cell lines, but is not expressed or expressed at significantly lower levels in malignant breast epithelium from human breast tumors and human breast tumor cell lines. To validate Per2's growth suppressive role in human breast cancer, we over-expressed human PER2 or the combination of Per2 and its dimerization partner Cry2 in MCF-7 human breast cancer cells and conducted cell proliferation assays. Cell proliferation assays, as shown in Figure 2, demonstrated that expression of Per2 alone, but not Cry2 alone induce a significant suppression in MCF-7 cell proliferation. However, when both Per2 and Cry2 were co-

expressed a very significant reduction in cell proliferation was observed compared to both vector controls and cells expressing either Per2 or Cry2 alone. Figure 3 demonstrates Per2 expression in cells transiently transfected with Per2 in these studies. These data clearly indicate that Per2 alone and in combination with Cry2 regulate cell proliferation and thus, likely interact to regulate breast tumor cell cycle.

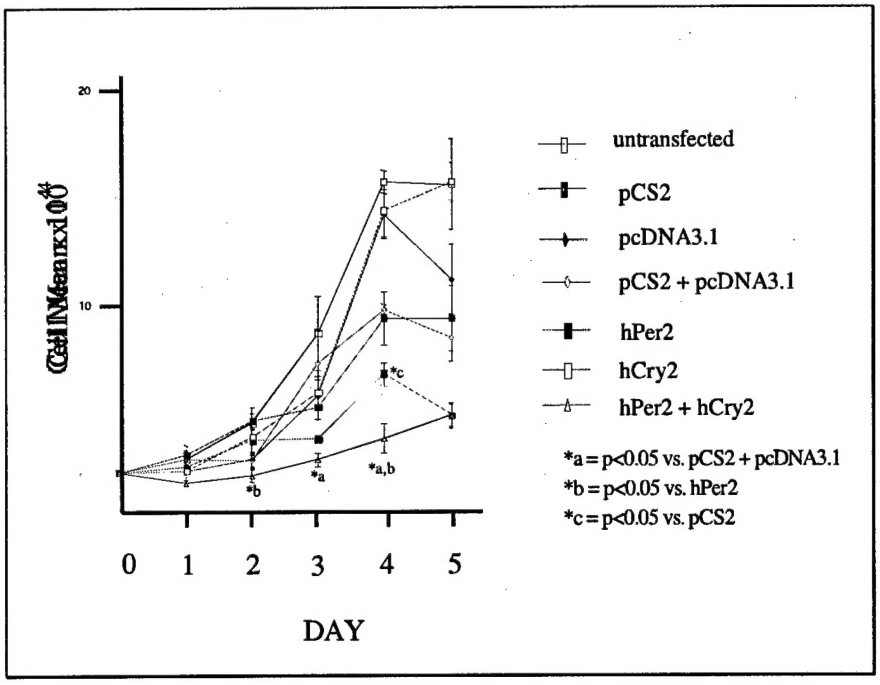


Figure 2. Effect of Per2 and Cry2 on the growth of MCF-7 cells. Cell proliferation assays were conducted on parental, vector-transfected, Per2-overexpressing MCF-7 cells, Cry2-overexpressiong MCF-7 cells, and cells overexpressing both Per2 and Cry2. Cells were counted on a hemacytometer using the trypan blue exclusion test for viable cells every day for five days. One-way ANOVA followed by a Newman-Kuels post-hoc test analysis were used to determine statistically significant differences in the cell number among different groups. (n = 3).

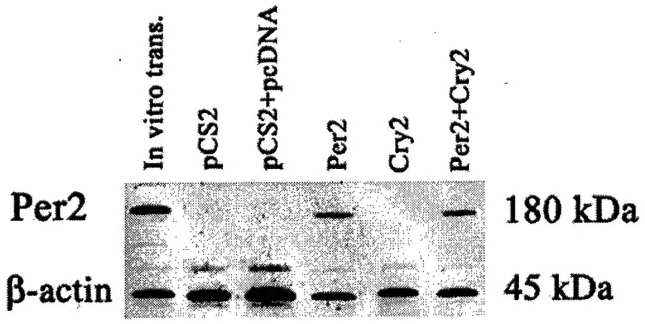


Figure 3. Confirmation of PER2 over-expression in MCF-7 cells. MCF-7 cells were transiently transfected with empty vectors, *per2* cDNA, or both *per2* and *cry2* cDNAs. After 48 hours, total cell extracts were prepared and subjected to Western blot analysis. Untransfected and empty vector-transfected cells showed no expression of PER2 whereas cells transfected with *per2* cDNA exhibited a robust expression of PER2 protein. Over-expression was also seen in cells transfected with both *per2* and *cry2* cDNA.

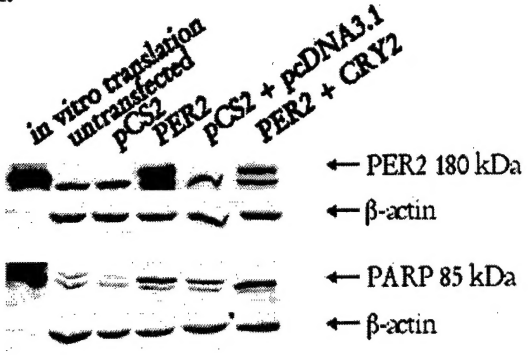
It is well established that various clock genes show an expression pattern that displays a circadian rhythm and is correlated with cell cycle progression in various cell types in culture. Furthermore, we have postulated that if clock genes can regulate circadian rhythms, they must in turn be able to regulate the cell cycle of these tissues. To examine the ability of the clock gene Per2 to regulate cell cycle asynchronously growing MCF-7 cells that were transiently transfected with empty vectors (pCS2 or pcDNA3.1), a vector expressing green fluorescent protein (GFP) or vectors expressing Per2 or Per2 and Cry2. Twenty four hours following transfection, cells were harvested and cell cycle phase distribution was assessed from propidium idodide-labeled cells by flow cytometry. As shown in Table 1 cells transfected with Per2 demonstrated a significant increase in the number of cells in G1 (82.2%) and an associated significant decrease of cells in S-phase (7.1%) compared to GFP expressing cells. Cells transfected with and expressing both Per2 and Cry2 showed 89% frequency in G1 and 0.58% in S-phase. Thus, it is clear that Per2 can block the progression of cells through the cell cycle at the G1/S border and that co-expression of Per2/Cry2 can enhance this effect.

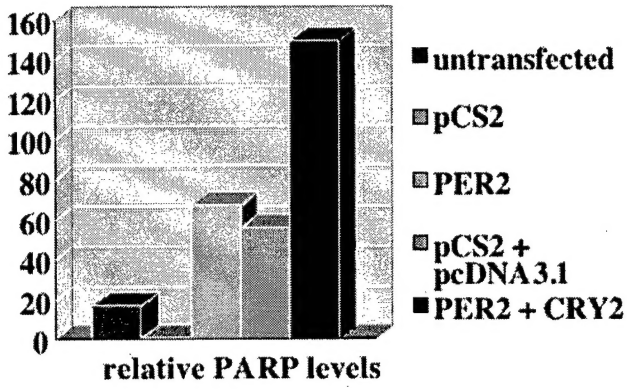
Table 1. Cell cycle analysis of breast cancer cell lines expressing Per2 or Cry2 genes. Asynchronously growing cell lines transiently transfected with GFP, empty vectors (pCS2, pcDNA3.1) or with Per2 or Per2 and Cry2 were plated at a density of 2 x 105 cells per plate and harvested 24 h following transfection. Cell cycle analysis was assessed in cells from each treatment group using propidium iodide-labeled cells and flow cytometry.

Treatment	G1	S	G2/M
GPF	64.14	15.57	15.29
pCS2	65.36	18.03	16.61
PER2	82.24*	7.16*	10.6
pCS2+pcDNA	62.61	17.49	19.90
PER2+CRY2	89.19*	0.58**	10.22

The blockade of MCF-7 cells transfected with and expressing Per2 at the G1/S border of the cell cycle suggests that many of these cells should either be induced to undergo cell death (apoptosis) or should show enhanced sensitivity to apoptotic stimuli. To determine if cells expressing elevated levels of Per2 showed increased apoptosis, we conduced PARP cleavage assays as well as by annexin V-FITC and propidium iodide double staining. Figure 4 is a Western blot analysis of both Per2 expression and expression of the 85 KDa PARP cleavage fragment.. As seen in this figure, in cells transfected with and expressing Per2 alone or Per2 in combination with Cry2, the expression of a p85 PARP cleavage fragment was significantly enhanced. These data supported by Annexin –FITC analysis (data not shown) demonstrate that expression of Per2 induces human breast cancer cell lines to undergo apoptosis. Furthermore, preliminary studies extending this work suggest that the addition of apoptotic stimuli (tamoxifen, or adriamycin) to MCF-7 cells expressing Per2 can potentiate the actions of Per2 on apoptosis.

A.





B.

Figure 4. Per2 over-expressing cells undergo apoptosis. A) Western blot analysis of MCF-7 cells were transiently transfected with empty vectors, *per2* cDNA, or both *per2* and *cry2* cDNA for PARP cleavage. After 48 hours, total cell extracts were prepared and subjected to Western blot analysis using an antibody directed against the 85 kDa cleaved fragment of PARP (poly (ADP-ribose) polymerase). PER2 over-expressing cells displayed a significant increase in cleaved PARP protein levels when compared to untransfected or empty vector transfected cells. These results show that over-expression of PER2 induces apoptosis. B) Densitometric analysis of PARP expression in Per2 treated cells. n=3. * $p < 0.05$ vs. controls.

<u>*Per 2 regulation of cyclins.*</u> Because Per2 over-expression arrests MCF-7 cells in G1, cell cycle regulators such as *cyclin D1* and *c-myc* expression should also be diminished in reponse to Per2 expression. The other cyclins of interest are more difficult to predict due to the fact there is nothing in the literature to direct my rationale. Nonetheless, *cyclin A* should also be downregulated since it is a regulator of G2 but *cyclin B* should be unaffected. Likewise, the expression of *mdm-2* should remain level as *Per2* knockout mice have not shown altered *mdm-2* mRNA expression. Figure 5 shows the densitometric analysis of Western blot analyses of *cyclin D1* expression in control MCF-7 cells as compared to MCF-7 cells transfected with and expressing Per2 or Per2 and Cry2. These studies demonstrate that Per2 expression either alone or in combination with Cry2 represses the expression of *cyclin D1*.

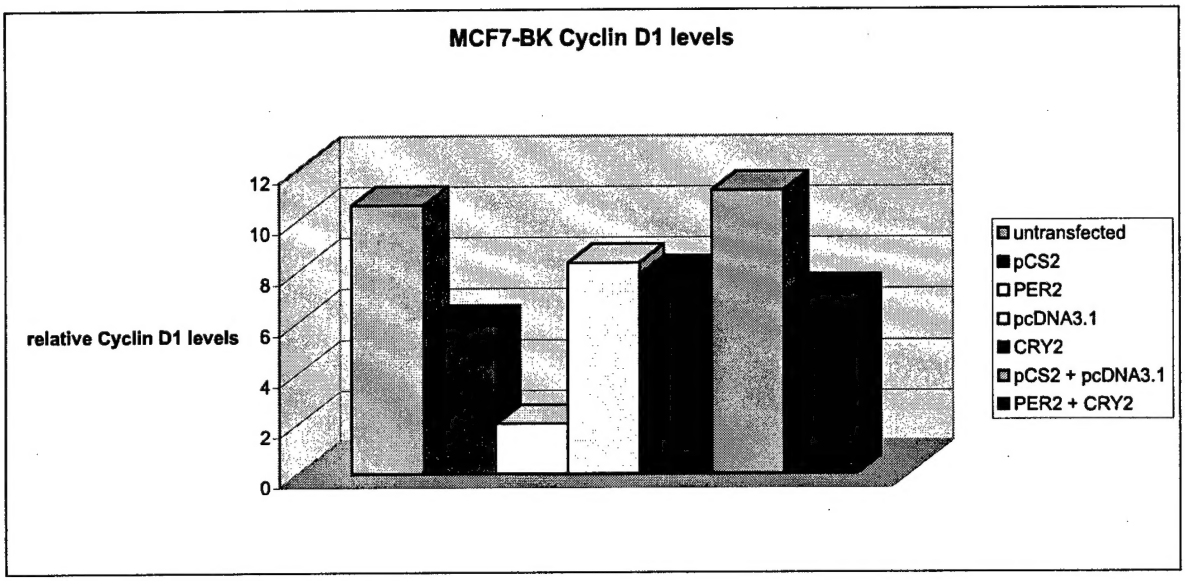


Figure 5. Cyclin D1 expression in response to Per2. A) Western blot analysis of MCF-7 cells were transiently transfected with empty vectors, *per2* cDNA, or both *per2* and *cry2* cDNA for cyclin D1. After 48 hours, total cell extracts were prepared and subjected to Western blot analysis using an antibody directed against human cyclin D1. PER2 over-expressing cells displayed a significant decrease in cyclin D1 protein levels when compared to untransfected or empty vector transfected cells. Interestingly, the co-expession of PER2 and CRY2 do not induce a significant decrease in cyclin D1 protein levels.

KEY RESEARCH ACCOMPLISHMENTS:

- Per2 mRNA is expressed in normal breast epithelial cells isolated from human breast tumor samples, but is not expressed or expressed at considerably lower levels in malignant breast tumor cells isolated from the same breast tumor samples. As well, human breast tumor cell lines expressed reduced Per2 mRNA as compared to non-malignant, immortalized tert-human breast epithelial cells.

- Expression of elevated levels of Per2 in MCF-7 human breast tumor cells significantly suppresses the proliferation of these cells, increases the number of cells in G1 of the cell cycle, apparently blocking the cell cycle at the G1/S border. In addition, enhanced expression of Per2 in MCF-7 breast tumor cells significantly increases the percentage of cell undergoing apoptosis.

- Associated with the G1/S blockade of the cell cycle in MCF-7 cells, with increased growth suppression and enhanced apoptosis in cells expressing high levels of Per2, is the suppression of the cell cycle regulatory proteins *cycling D1* and *c-myc*.

REPORTABLE OUTCOMES:

Army/DoD Breast Cancer Program report
Abstract 2005 Era of Hope Meeting
Manuscript in Preparation

CONCLUSIONS: The major questions addressed in this project is whether the clock gene Per2 is expressed in both normal and malignant breast epithelial cells, if Per2 expression functions to inhibit breast tumor growth and if Per2 expression can regulate the progression of the cell cycle in breast tumor cells *in vitro*. The initial phase of our project involved examining Per2 mRNA expression from primary breast tumors using a LCM approach to isolate both normal and malignant breast epithelial cells from the same sample. Using this approach and RNA isolated from human breast tumor cell lines, we found the Per2 mRNA is expressed in normal breast epithelial cell lines, but is either not expressed or is expressed at considerably lower levels in breast tumor cells. When we transfected MCF-7 human breast tumor cells and expressed elevated levels of Per2, breast tumor cell proliferation was significantly inhibited. Furthermore, an even greater degree of growth-inhibition was observed when Per2 was co-expressed with it dimerization partner Cry2. Correlative studies examining the effect of Per2 or Per2 in combination with Cry2 demonstrated that expression of Per2 or Per2/Cry2 induced a blockade of cell cycle progression at the G1/S border. This blockade at the G1/S border by Per2 was also associated with a significant increase in the number of breast tumor cells undergoing apoptosis. In addition, we hypothesize that cells expressing elevated levels of Per2 should show an enhanced sensitivity to apoptotic stimuli such as removal of estrogen, antiestrogen-treatment or even chemotherapeutic agents. In addition to these studies, we have also demonstrated that the cell cycle regulatory protein cyclin D1 and c-myc were down-regulated in the presence of Per2 expression and thus, may play an important role in mediating Per2's regulation of cell cycle progression and breast tumor cell apoptosis. From these studies, we conclude that Per2 is a tumor suppressor gene whose expression and/or function is significantly diminished in breast tumor cells.

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